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The Extracellular Signal-regulated Kinase Pathway Regulates the Phosphorylation of 4E-BP1 at Multiple Sites*

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The phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent stimulator of Erk, leads to the phosphorylation of 4E-BP1 and its dissociation from eIF4E. In contrast to agonists such as insulin, this occurs independently of PKB activation. In this report, we investigate the mechanism by which TPA regulates 4E-BP1 phosphorylation. Treatment of HEK293 cells with TPA was found to result in the phosphorylation of 4E-BP1 at Ser^{64}, Thr^{69}, and Thr$^{36/45}$. The TPA-stimulated phosphorylation of all these sites is sensitive to inhibitors of MEK and to the inhibitor of mTOR, rapamycin, indicating that inputs from both mTOR and MEK are required for the regulation of 4E-BP1 phosphorylation by TPA. Indeed, evidence is presented that mTOR may initially be required for the phosphorylation of Thr^{45} in a priming step, which is necessary for the subsequent phosphorylation of Ser^{64} and Thr^{69} through an Erk-dependent pathway. Overexpression of constitutively active MEK in HEK293 cells resulted both in the phosphorylation of 4E-BP1 at Ser^{64} and Thr$^{36/45}$ and its release from eIF4E. In this case, the phosphorylation of these sites was also blocked by inhibitors of MEK or by rapamycin. In conclusion, the Erk pathway, via mechanisms also requiring mTOR, regulates the phosphorylation of multiple sites in 4E-BP1 *in vivo* and this is sufficient for the release of 4E-BP1 from eIF4E.

mRNA translation is conventionally divided into three stages: initiation, elongation, and termination. Of these, initiation is considered the rate-limiting step and therefore is considered the most important step in the acute regulation of translation. Essential for initiation of cap-dependent protein synthesis is the assembly of the initiation complex, eukaryotic initiation factor (eIF)[1] 4F, containing the initiation factors eIF4G, a large scaffolding protein, eIF4E, the protein which binds to the 5′ cap structure and eIF4A, a bidirectional RNA helicase (for review, see Ref. 1). The formation of the eIF4F complex is regulated by a variety of stimuli through the activation of multiple signal transduction pathways. eIF4E-binding protein-1 (4E-BP1), also known as protein-heat and acid-stable I, is a translational repressor that regulates eIF4F assembly and therefore cap-dependent translation (for review, see Ref. 1). 4E-BP1 binds to a site on eIF4E that overlaps the binding site for eIF4G and it therefore competes with eIF4G for binding to eIF4E, which results in the inhibition of eIF4F assembly (2–4). The binding of 4E-BP1 to eIF4E is regulated through the phosphorylation of 4E-BP1. Phosphorylation of 4E-BP1 leads to a reduction in its affinity for eIF4E and its subsequent dissociation from eIF4E (2, 5–8).

Five phosphorylation sites each conforming to a (S/T)P motif have been identified in 4E-BP1; Thr^{36}, Thr^{45}, Ser^{64}, Thr^{69}, and Ser^{82} (based on rat sequence, +1 for human (9–11)). The phosphorylation of 4E-BP1 at Thr^{45} and Ser^{64}, sites adjacent to the conserved eIF4E-binding motif, is thought to be the most important in influencing the binding of 4E-BP1 to eIF4E (12, 13). However, each site differentially influences the binding of 4E-BP1 to eIF4E and mRNA translation by affecting either the affinity of 4E-BP1 for eIF4E or (indirectly) influencing the phosphorylation of other sites (12, 14).

The signaling pathways leading to insulin-induced increases in the phosphorylation of 4E-BP1 have been the focus of much attention. Insulin is thought to regulate the phosphorylation of 4E-BP1 through the phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway (15) and this is thought to be dependent on both the phosphorylation and activation of PKB (16, 17). *In vitro*, 4E-BP1 is an excellent substrate for Erk, which phosphorylates all five sites, albeit with differing efficiencies (9). Ser^{64} is the major *in vitro* Erk phosphorylation site and is also a major site of phosphorylation in response to insulin *in vivo* (6, 9). However, *in vivo*, the activation of Erk appears neither necessary nor sufficient for the phosphorylation of 4E-BP1 in response to insulin (15, 18, 19).

Rapamycin, a specific inhibitor of mTOR, blocks the effects of insulin or phorbol ester on the phosphorylation of 4E-BP1 (7, 18, 19). *In vitro*, immunoprecipitated mTOR can phosphorylate 4E-BP1 at Thr^{36}, Thr^{45}, Thr^{69}, Ser^{64}, and Ser^{82}, albeit with low and differing efficiencies (12, 20, 21).

We have previously shown that, in HEK 293 cells, the phorbol ester TPA stimulates the phosphorylation of 4E-BP1 via mechanisms that are independent of PI 3-kinase and PKB (19). The phosphorylation and regulation of 4E-BP1 by TPA are inhibited by the MEK inhibitors PD098059 and U0126 indicating that the Erk pathway plays a role in the regulation of 4E-BP1 by TPA (19). In contrast, the regulation of 4E-BP1 by insulin in these cells is independent of MEK. In addition, there is an increasing number of other reports, using a variety of

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[1] The abbreviations used are: eIF, eukaryotic initiation factor; HEK, human embryonic kidney; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 4E-BP1, eIF4E binding protein 1; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; Erk, extracellular signal-regulated kinase; PKC, protein kinase C; BIM, bisindolylmaleimide; CA-MEK, constitutively active mitogen-activated protein kinase/extracellular signal-regulated kinase.

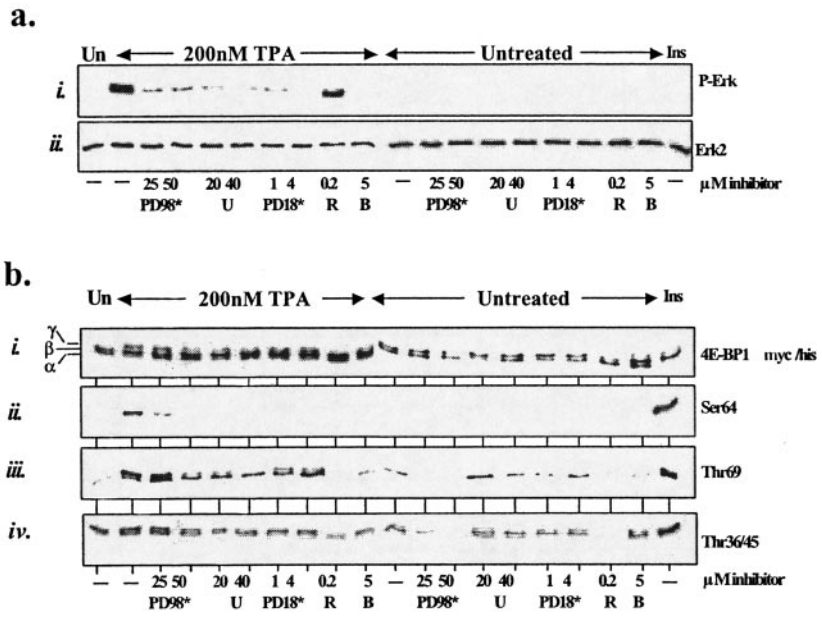

FIG. 1. **TPA stimulates 4E-BP1 phosphorylation through the MAP kinase pathway.** HEK293 cells transiently transfected with pCDNA3– 4E-BP1 were serum starved for 18 h. Cells were pretreated with the indicated inhibitors (*PD98**, PD098059; *U*, U0126; *PD18**, PD184352; *r* rapamycin; *B,* BIM) for 45 min prior to the addition of 1 μM TPA or 100 nM insulin (*ins*) for 1 h (or left untreated (*Un*)). *a,* samples of cell lysates (20 μg) were applied to a SDS-polyacrylamide gel followed by Western blotting using: (i) anti-phospho Erk and (ii) anti-Erk2 antisera. *b,* samples of cell lysates (20 μg) were run on a SDS-polyacrylamide gel followed by Western blotting using: (i) anti-Myc antibody or (ii-iv) phospho-specific antibodies against 4E-BP1. The positions of the three forms of 4E-BP1 separated in this gel system (α, β, and, γ in order of increasing state of phosphorylation) are indicated. (ii) anti-phospho Ser^{64}; (iii) anti-phospho Thr^{69}; and (iv) anti-phospho Thr*36/45* antisera. Similar data were obtained from three separate experiments.

physiological stimuli, indicating a role for Erk pathway in the phosphorylation of 4E-BP1 (22, 23).

In this report we demonstrate that TPA, a potent activator of Erk, leads to the phosphorylation of 4E-BP1 at $Thr^{36/45}$, Ser^{64}, and Thr^{69}, and that this leads to the dissociation of eIF4E from 4E-BP1. Phosphorylation at these sites is sensitive to inhibitors of MEK and rapamycin, indicating that inputs from both mTOR and MEK are required for TPA regulation of 4E-BP1 phosphorylation. Additionally, activation of the Erk pathway in HEK293 cells through the overexpression of constitutively active MEK resulted in the phosphorylation of 4E-BP1 at positions $Thr^{36/45}$ and Ser^{64}, and its release from eIF4E.

MATERIALS AND METHODS

Chemicals and Materials—Microcystin LR, bisindolylmaleimide (BIM), rapamycin, and PD098059 were purchased from Calbiochem. Materials for tissue culture were from Invitrogen. m^7GTP-Sepharose was from Amersham Biosciences, Inc. U0126 was obtained from Promega. All other chemicals (unless stated) were obtained from Sigma. PD184352 was provided by the Division of Signal Transduction Therapy, Dundee University.

Cell Culture and Treatment of Cells—HEK293 cells were maintained in Dulbecco's modified Eagle's media (Invitrogen) supplemented with 10% fetal calf serum. Prior to treatment, cells were grown to 80% confluence before being serum starved for 18 h. Details of treatments are provided in the figure legends. After treatment, cells were washed in phosphate-buffered saline and lysed in extraction buffer (0.1% Triton X-100, 50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 1 mM benzamidine-HCl, 0.5 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of pepstatin A, antipain, and leupeptin). The lysates were then centrifuged for 10 min at 16,000 × *g*. The supernatants were removed and used for further analysis.

SDS-PAGE and Immunoblotting—SDS-PAGE and Western blotting were performed as described previously (24). Anti-human eIF4E antiserum was raised against a synthetic peptide corresponding to residues 5–23 of the protein. The anti-4E-BP1 antibody was a generous gift from Adri Thomas (Utrecht). Anti-phospho 4E-BP1, PKB, and Erk antibodies were supplied by New England Biolabs. Anti-Erk5 antibodies were obtained from Upstate Biotech. Densitometry was performed using the program Image Quant (Amersham Biosciences, Inc.).

m^7GTP-Sepharose Chromatography—m^7GTP-Sepharose CL-6B (Amersham Biosciences, Inc.) was added to 0.2–0.5 mg of cell lysate. The lysates were then mixed with the beads for 1 h at 4 °C. The m^7GTP-Sepharose was pelleted by centrifugation at 1000 × *g* for 2 min. The beads were then washed three times in extraction buffer. For SDS-PAGE, proteins were removed from the m^7GTP matrix by boiling in SDS loading buffer and, after centrifugation, the supernatant was loaded on to the gel.

Plasmids—A PCR directed fragment-encoding rat 4E-BP1 was digested with *Hin*dIII and *Bam*HI and cloned into pcDNA3.1myc/3his⁻ (Invitrogen) to generate pcDNA3–4E-BP1 expressing Myc and His-tagged 4E-BP1. The codon for residue Thr^{45} of 4E-BP1 within pcDNA3–4E-BP1 was mutated to encode alanine using the QuikChange™ mutagenesis kit (Stratagene) producing the vector pcDNA3–4E-BP1T45A. pCA-MEK expressing a constitutively active form of MEK1 (ΔN3-S218E/S222D MEK1), and pErk2-MEK-La expressing a constitutively active form of Erk (25), were kindly provided by Drs. Axel Knebel (University of Dundee) and Melanie Cobb (University of Texas, Southwestern Medical Center, Dallas), respectively.

Transient Transfections—Transient transfections were performed as described by Alessi *et al.* (26).

RESULTS

The Phorbol Ester, TPA, Leads to the Phosphorylation of 4E-BP1 through the Erk Pathway—We have previously provided evidence that the phorbol ester, TPA elicits the phosphorylation of 4E-BP1 though a mechanism that is dependent upon MEK but independent of PI 3-kinase and PKB (19). To characterize further the role of the MEK/Erk pathway in the phosphorylation of 4E-BP1, we first investigated which sites within 4E-BP1 were phosphorylated in response to TPA using previously described phospho-specific antibodies (12).

To examine the phosphorylation state of 4E-BP1, Myc/His-tagged 4E-BP1 (4E-BP1myc/his) was expressed in HEK293 cells. The cells were then treated with TPA, insulin, and/or specific kinase inhibitors. As previously observed, TPA treatment led to the phosphorylation of Erk (Fig. 1*a*) and a decrease in the mobility of 4E-BP1 (Fig. 1*b, i*) on SDS-PAGE, which is indicative of its phosphorylation. TPA caused an increase in the phosphorylation of 4E-BP1myc/his at Ser^{64} and Thr^{69} and a small increase in the phosphorylation of $Thr^{36/45}$ as assessed using anti-phospho-$Ser(P)^{64}$, $Thr(P)^{69}$, and $Thr(P)^{36/45}$ specific antibodies (Fig. 1*b, ii-iv*). Similar increases in the phosphorylation of 4E-BP1myc/his were detected when cells were stimulated with insulin (Fig. 1*b*). A basal level of phosphorylation of 4E-BP1 at both Thr^{69} and $Thr^{36/45}$ was also detected in serum-starved untreated cells (Fig. 1*b, iii* and *iv*).

Pretreatment of the cells with each of three structurally distinct inhibitors of MEK (the upstream activator of Erk), PD098059, U0126, and PD184352 (27–29), resulted in a dose-dependent inhibition of TPA-stimulated Erk phosphorylation as assessed using an anti-phospho-Erk antibody (Fig. 1*a*, *i*) (as a loading control the same samples were probed with anti-Erk 2 antibody (Fig. 1*a*, *ii*)). All three MEK inhibitors caused an inhibition of TPA-stimulated 4E-BP1 phosphorylation at

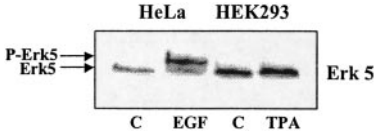

FIG. 2. **TPA does not cause the phosphorylation of Erk5.** HEK293 or HeLa cells were serum starved for 18 h. Cells were treated with either TPA or epidermal growth factor (*EGF*) as indicated. 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-Erk5 antiserum.

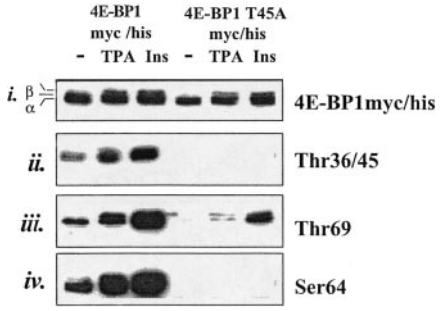

FIG. 3. **TPA stimulated phosphorylation of 4E-BP1 at Thr^{36}, Ser^{64}, and Thr^{69} requires phosphorylation at Thr^{45}.** HEK293 cells transiently transfected with pcDNA3–4E-BP1 and pcDNA3–4E-BP1T-45A were serum starved for 18 h. Cells were treated with either TPA or insulin (Ins) for 1 h. 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using: (i) anti-Myc, and (ii-iv) phospho-specific antibodies for 4E-BP1. The positions of the three forms of 4E-BP1 separated in this gel system are indicated (α, β, and γ in order of increasing state of phosphorylation). Similar data were obtained from three separate experiments.

Ser^{64} and partially blocked the phosphorylation at Thr^{69} and Thr$^{36/45}$. These inhibitors also decreased the basal phosphorylation of 4E-BP1 at Thr^{69} and Thr$^{36/45}$ (Fig. 1*b*, *iii* and *iv*).

It has been reported that each of the three MEK inhibitors used in this study can also inhibit MEK5, the upstream activator of Erk5 (30, 31). Thus, it was possible that the effect of these inhibitors was through inhibition of Erk5. We therefore investigated whether TPA could cause phosphorylation of Erk5 in HEK293 cells. HEK293 cells were treated with TPA and extracts were analyzed by SDS-PAGE and immunoblotting using an anti-Erk5 antibody (Fig. 2). As a positive control, HeLa cell lysates treated with EGF were run alongside (32). EGF treatment of HeLa cells leads to the phosphorylation of Erk5 as indicated by a shift in its mobility on SDS-PAGE. However, no phosphorylation of Erk5 was detected upon TPA stimulation of HEK293 cells (Fig. 2), thus excluding a role for this pathway in the effect of TPA on 4E-BP1.

Pretreatment of HEK293 cells with rapamycin, inhibited the TPA-stimulated phosphorylation of 4E-BP1 at Ser^{64} and abolished or markedly inhibited both basal and its TPA-stimulated phosphorylation at Thr^{69} and Thr$^{36/45}$, respectively (Fig. 1*b*). However, rapamycin had no effect on the phosphorylation of Erk (Fig. 1*a*), suggesting that it exerts its effect independently of the MEK/Erk pathway.

TPA activates both classical and novel PKC isoforms (33). Consistent with this, pretreatment of cells with the broad spectrum PKC inhibitor BIM inhibited the effect of TPA on the phosphorylation of both Erk and 4E-BP1myc/his (Fig. 1, *a* and *b*).

These results provide strong evidence that the Erk pathway (specifically MEK) plays a role in the phosphorylation of 4E-BP1myc/his at Ser^{64}, Thr^{69}, and Thr$^{36/45}$ induced by TPA.

TPA Stimulated Phosphorylation of 4E-BP1 at Thr^{36}, Ser^{64}, and Thr^{69} Requires Phosphorylation at Thr^{45}—It has previously been reported that the extent of insulin-stimulated phosphorylation of 4E-BP1 at one site can modulate the phosphorylation of another site, with Thr^{36} or Thr^{45} having the most dramatic influence on the phosphorylation at other sites (12, 14, 34).

To investigate whether TPA-stimulated 4E-BP1 phosphorylation was regulated similarly, Thr^{45} of 4E-BP1 was mutated to an alanine creating the vector 4E-BP1T45A Myc/His. The phosphorylation of overexpressed 4E-BP1T45A Myc/His and 4E-BP1myc/his upon insulin or TPA stimulation was monitored using the phospho-specific antibodies. As previously shown (see Fig. 1), stimulation of cells with either TPA or insulin resulted in the phosphorylation of 4E-BP1myc/his at Thr$^{36/45}$, Ser^{64}, and Thr^{69} (Fig. 3). In contrast, neither TPA nor insulin led to the phosphorylation of 4E-BP1T45A Myc/His at Thr^{36} or Ser^{64} and there was a severe reduction in the phosphorylation at Thr^{69} (Fig. 3). This suggests that phosphorylation of these residues in 4E-BP1 in response to TPA depends upon (prior) phosphorylation of 4E-BP1 at Thr^{45}, as suggested earlier for the phosphorylation of 4E-BP1 in response to insulin or serum (34, 35).

Transient Transfection of Constitutively Active MEK Leads to the Phosphorylation of 4E-BP1—To demonstrate directly that MEK plays a role in the phosphorylation of 4E-BP1, HEK293 cells were transiently co-transfected with the vector pCA-MEK encoding constitutively active MEK (CA-MEK) together with pcDNA3–4E-BP1 encoding 4E-BP1myc/his. As a control, cells were co-transfected with pcDNA3–4E-BP1 and an empty vector. The phosphorylation of 4E-BP1myc/his was investigated using phospho-specific antibodies for Thr$^{36/45}$, Ser^{64}, and Thr^{69}. Overexpression of CA-MEK led to the phosphorylation of Erk and a decrease in the mobility of 4E-BP1myc/his on SDS-PAGE, indicative of increased phosphorylation (Fig. 4, *a* and *b, i*). Overexpression of CA-MEK resulted in the phosphorylation of Ser^{64} (Fig. 4*b, ii*) and increased phosphorylation at Thr$^{36/45}$ as assessed using phospho-specific antibodies (Fig. 4*b, iii*). In contrast to the situation for cells treated with TPA, no significant change in the phosphorylation of Thr^{69} was detected. However, some variation between experiments in the basal/control levels of phosphorylation at Thr$^{36/45}$ and Thr^{69} was observed. Treatment of cells overexpressing CA-MEK and 4E-BP1myc/his with U0126, PD098059, or PD184352, inhibitors of MEK (27, 28), resulted in inhibition of the phosphorylation of 4E-BP1myc/his at Thr$^{36/45}$ and Ser^{64} in parallel with the inhibition of Erk phosphorylation. In general, U0126 was more effective than PD098059 at inhibiting the phosphorylation of both Erk and 4E-BP1myc/his. This most likely reflects differences in the mechanisms by which these two inhibitors work. U0126 is thought to block the activity of MEK whereas PD098059 is thought to inhibit MEK activation (27, 28, 36).

Incubation of cells expressing CA-MEK with rapamycin, an inhibitor of mTOR, also inhibited CA-MEK-induced phosphorylation of 4E-BP1 at Ser^{64} and abolished or markedly inhibited both basal and MEK-stimulated phosphorylation at Thr$^{36/45}$ and Thr^{69}, respectively, without affecting the phosphorylation of Erk (Fig. 4, *a* and *b*).

The activation of MEK may lead to the activation of various PKC isoforms including PKCδ, which itself has been implicated in the phosphorylation of 4E-BP1 (37). To investigate whether MEK was acting through PKC, cells expressing CA-MEK were treated with the PKC inhibitor BIM. In contrast to the effect of BIM on TPA stimulated phosphorylation of 4E-BP1 (Fig. 1), BIM had only a small effect on the phosphorylation of 4E-BP1 at the sites investigated (Fig. 4, *a* and *b*), indicating that PKC was probably not mediating the effect of CA-MEK.

To rule out the possibility that MEK activation could lead to the phosphorylation of 4E-BP1 through activation of PKB, the phosphorylation status of PKB in cells expressing constitutively active MEK was monitored using a phospho-specific an-

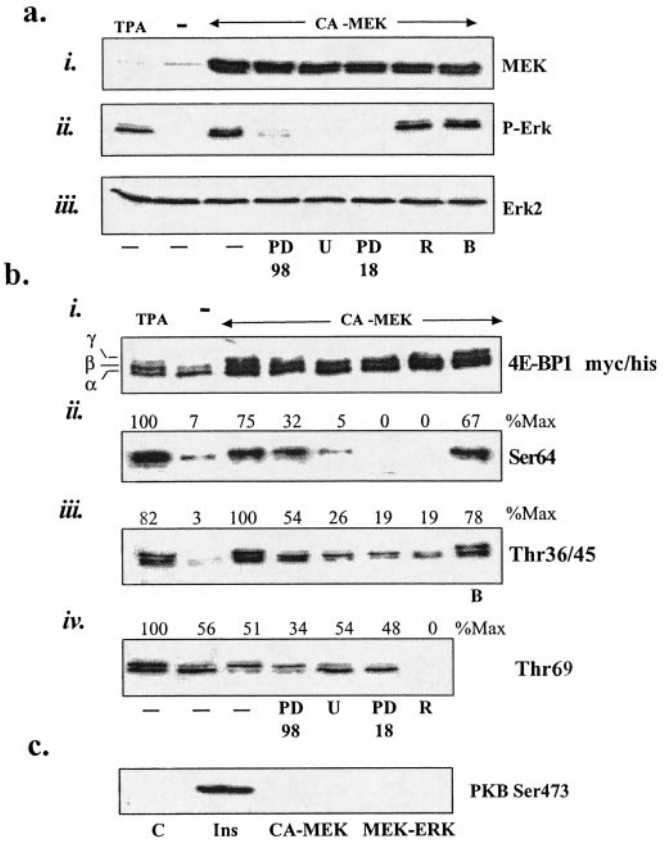

FIG. 4. **Constitutively active MEK regulates 4E-BP1 phosphorylation *in vivo*.** HEK293 cells transiently transfected with pCA-MEK and pcDNA3–4E-BP1 were serum starved for 18 h. Cells were treated with the inhibitors indicated for 1 h (*PD98*, PD098059; *U,* U0126; *PD18*, PD184352; *r,* rapamycin; *B,* BIM). *a,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using: (i) anti-MEK, (ii) anti-phospho-Erk, or (iii) anti-Erk2 antisera. *b,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using: (i) anti-Myc to detect 4E-BP1myc/his. The positions of the three forms of 4E-BP1 separated in this gel system (α, β, and, γ in order of increasing state of phosphorylation) are indicated. (ii) anti-phospho Ser^{64}; (iii) anti-phospho Thr$^{36/45}$; and (iv) anti-phospho-Thr^{69} antisera. *c,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-phospho-PKB (Ser^{473}). Similar data were obtained from three separate experiments. The change in phosphorylation is expressed as percentage of maximal phosphorylation are above blots b (*ii-iv*).

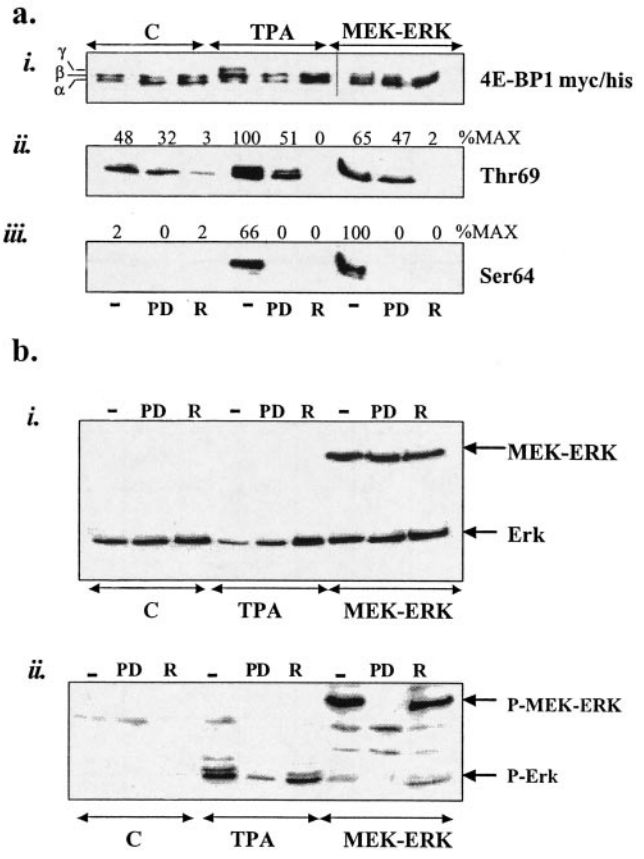

FIG. 5. **Constitutively active Erk regulates 4E-BP1 phosphorylation *in vivo*.** (*a*) HEK-293 cells transiently transfected with pcDNA3–4E-BP1 or pcDNA3–4E-BP1 and pERK2-MEK1-LA were serum starved for 18 h. Cells transfected with pcDNA3–4E-BP1 were preincubated for 45 min with either 50 μM PD098059 (*PD*) or 200 nM rapamycin (*R*) prior to the treatment with 1 μM TPA. Cells transiently transfected with pcDNA3–4E-BP1 or pcDNA3–4E-BP1 and pERK2-MEK1-LA were treated with 50 μM PD098059 (*PD*) or 200 nM rapamycin (*R*) for 1 h. 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using *a,* (i) anti-Myc to detect 4E-BP1myc/his. The positions of the three forms of 4E-BP1 separated in this gel system (α, β, and γ in order of increasing state of phosphorylation) are indicated. (ii) Anti-phospho-Thr^{69}. (iii) Anti-phospho Ser^{64}. *b,* (i) Anti-Erk. (ii) Anti-phospho Erk. These results are representative of three separate experiments. The quantification of phosphorylation, expressed as percentage of maximal phosphorylation, is indicated above blots *a* (ii and iii).

tibody against Ser^{473}. Phosphorylation of this site is required for activation of PKB (26). As a positive control, cells were treated with insulin. Insulin treatment resulted in a large increase in the phosphorylation of PKB at Ser^{473}. In contrast, expression of constitutively active MEK did not (Fig. 4*c*). Thus, CA-MEK does not activate PKB. These results provide evidence that MEK activation leads to the phosphorylation of 4E-BP1myc/his *in vivo* at Ser^{64} and Thr$^{36/45}$ through an mTOR-dependent mechanism, which does not involve activation of PKB.

Transient Transfection of Constitutively Active Erk Results in the Phosphorylation of 4E-BP1—As an adjunct to the above and to define the role of Erk (as opposed to MEK) in the phosphorylation of 4E-BP1, HEK293 cells were transiently co-transfected with pcDNA3–4E-BP1 expressing 4E-BP1myc/his and pERK2-MEK1-LA expressing a MEK/ERK fusion protein which has constitutive Erk activity (25). As a control, cells were co-transfected with pcDNA3–4E-BP1 and an empty vector. The phosphorylation of 4E-BP1 was investigated using the phospho-specific antibodies for Ser^{64} and Thr^{69}. Expression of constitutively active MEK/Erk led to the phosphorylation of 4E-BP1myc/his at Ser^{64} and a shift in its mobility on SDS-PAGE (Fig. 5). In contrast to cells stimulated with TPA, a small increase in the phosphorylation of Ser^{69} was detected upon expression of MEK-Erk. However, some variation in the basal/control levels of Thr^{69} phosphorylation was observed between experiments.

Pretreatment of the cells with PD098059, an inhibitor of MEK (27, 28), resulted in the inhibition the phosphorylation of Ser^{65} and a partial inhibition in the basal phosphorylation of Ser^{69} in MEK-Erk expressing cells. This was in parallel with the inhibition of Erk phosphorylation as assessed using a phospho-specific antibody to Erk. As a loading control, identical samples were probed with anti-Erk 2 antibody. Incubation of these cells with rapamycin also inhibited Erk-stimulated and basal phosphorylation of 4E-BP1myc/his at Ser^{64} and Thr^{69}, respectively. These results provide further, more direct, evidence that Erk activation, rather than any possible consequences of MEK activation, leads to the phosphorylation of 4E-BP1 *in vivo*.

Activation of the Erk Pathway Leads to the Release of 4E-BP1 from eIF4E—We have previously demonstrated that stimulation of HEK293 cells with TPA leads to the phosphorylation of

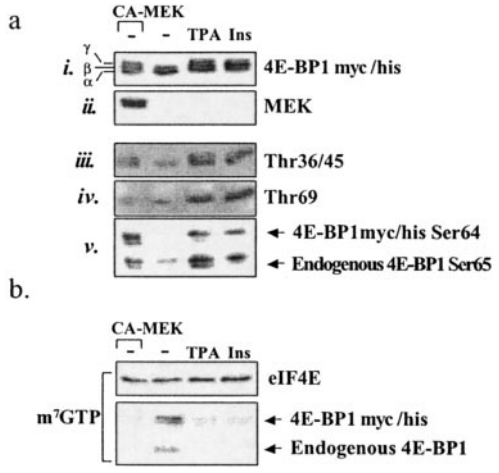

FIG. 6. **Role of the Erk pathway in the release of 4E-BP1 from eIF4E.** HEK293 cells transiently transfected with pCA-MEK and pcDNA3–4E-BP1 or pcDNA3–4E-BP1 were serum starved for 18 h. Cells were either left untreated (−), or treated with 100 nM insulin (*Ins*) or 1 μM TPA where indicated for 1 h. *a,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using (i) anti-Myc to detect 4E-BP1myc/his. The positions of the three forms of 4E-BP1 separated in this gel system (α, β, and γ in order of increasing state of phosphorylation) are indicated and (ii) anti-MEK antiserum. (iii) Anti-phospho-Thr$^{36/45}$. (iv) Anti-phospho-Thr^{69} antibodies or (v) anti-phospho-Ser^{64}. *b,* analysis of proteins isolated by m^{7}GTP-Sepharose pull down. Proteins were separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against either eIF4E or 4E-BP1.

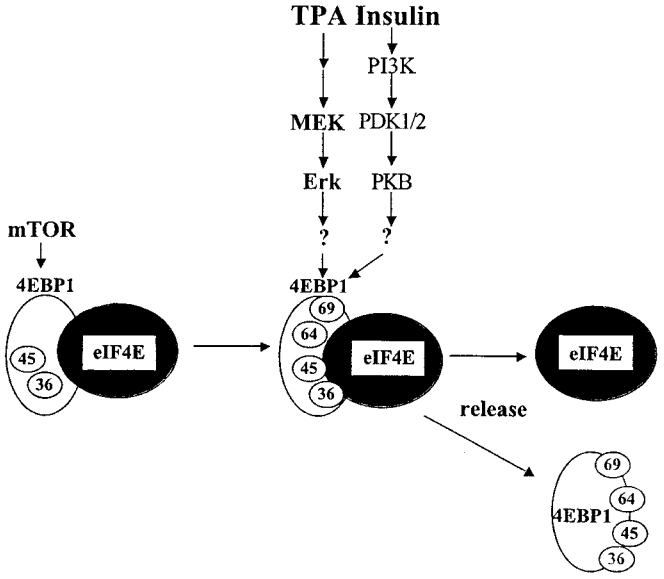

FIG. 7. **Diagram showing the possible mechanism and signaling pathways leading to the phosphorylation of 4E-BP1.** A two-step mechanism may be required for TPA stimulated 4E-BP1 phosphorylation. First, mTOR activity is required for the phosphorylation of Thr$^{36/45}$, which acts as a priming step for the subsequent phosphorylation of Ser^{64} and Thr^{69} in response to insulin or TPA through the PI 3-kinase or Erk pathways, respectively.

4E-BP1 and its dissociation from eIF4E and that these effects were blocked by inhibitors of MEK (19). To examine the role of the Erk pathway in the binding of 4E-BP1 to eIF4E, serum-starved HEK293 cells expressing 4E-BP1myc/his were treated with TPA, insulin, or transfected with CA-MEK and the association of eIF4E with 4E-BP1 was studied using m^{7}GTP-Sepharose chromatography. TPA, insulin, or the overexpression of CA-MEK led to the phosphorylation of 4E-BP1 (Fig. 6*a*) and to the dissociation of both endogenous and 4E-BP1myc/his from eIF4E (Fig. 6*b*). This indicates that the phosphorylation of 4E-BP1 through the activation of the Erk pathway is sufficient for release of 4E-BP1 from eIF4E.

DISCUSSION

We have previously demonstrated that in HEK293 cells, TPA causes the phosphorylation of 4E-BP1 and its dissociation from eIF4E via a mechanism which is independent of PI 3-kinase and PKB, but dependent on MEK (19). In this report we further investigate the phosphorylation of 4E-BP1 by TPA by determining the sites at which 4E-BP1 is phosphorylated under these conditions and identifying the signaling pathways which lead to 4E-BP1 phosphorylation. Treatment of HEK293 cells with TPA, a potent activator of Erk, results in the phosphorylation of 4E-BP1 at Thr^{69}, Ser^{64}, and Thr$^{36/45}$ (Figs. 1, 3, and 6). Phosphorylation at these sites is blocked by three structurally distinct inhibitors of MEK, PD098059, U0126, and PD154352 (Fig. 1). Additionally, expression of constitutively active MEK results in the phosphorylation of 4E-BP1 at Ser^{64} and Thr$^{36/45}$ (Fig. 4). TPA and the expression of an activated form of MEK each led to the dissociation of 4E-BP1 from eIF4E (Fig. 6). Taken together, this provides strong evidence that the Erk pathway regulates the phosphorylation and function of 4E-BP1 *in vivo*. In contrast to cells stimulated with TPA, expression of an activated form of MEK did not lead to a significant increase in the phosphorylation of 4E-BP1 at Thr^{69}. However, TPA stimulated phosphorylation of 4E-BP1 at Thr^{69} was sensitive to inhibitors of MEK. One possible explanation for this apparent discrepancy is that the phosphorylation of Thr^{69} requires both the activation of Erk and an additional input provided by TPA, which may activate multiple downstream pathways.

The phosphorylation of 4E-BP1 in response to TPA, constitutively active MEK or Erk is blocked by rapamycin, a specific inhibitor of mTOR (Figs. 1, 4, and 5). Interestingly, rapamycin also inhibited the basal phosphorylation of 4E-BP1 at Thr$^{36/45}$ (Figs. 1, 4, and 5). It has previously been demonstrated that serum-stimulated phosphorylation of Thr^{36} or Thr^{45} is necessary for the subsequent phosphorylation at Ser^{64} and Thr^{69} (34, 35). In this study we demonstrate that a Thr^{45} to Ala 4E-BP1 mutant does not undergo TPA-induced phosphorylation at Ser^{64} or Thr^{69} (Fig. 3). This indicates that the phosphorylation of Thr^{45} may be required for the subsequent phosphorylation of Ser^{64} and Thr^{69}. It is likely that mTOR, or an mTOR-dependent kinase, phosphorylates 4E-BP1 at Thr$^{36/45}$, as these sites are sensitive to rapamycin *in vivo*. Additionally, mTOR has been shown to phosphorylate these sites *in vitro* (12, 20). As Thr^{45} is required for the phosphorylation of Ser^{64} and Thr^{69} it would appear that phosphorylation by TPA may occur by a similar two-step mechanism as it has been proposed for serum-stimulated phosphorylation of 4E-BP1 (14, 34) (Fig. 7).

Erk is known to phosphorylate 4E-BP1 readily *in vitro* (6, 9, 18). However, Erk is unlikely to directly phosphorylate 4E-BP1 *in vivo* as Erk cannot phosphorylate 4E-BP1 bound to eIF4E (18, 38). PKCδ has been reported to phosphorylate 4E-BP1 *in vivo* (37). However, PKCδ or the conventional PKC isoforms probably do not play a role in MEK-induced phosphorylation of 4E-BP1 at the sites investigated, as BIM had a small effect on the MEK-induced phosphorylation of 4E-BP1 (Fig. 4). Therefore, 4E-BP1 phosphorylation induced by TPA or activated forms of MEK or Erk is likely to be through the activation of an as yet unidentified kinase or kinases that are regulated through MEK/Erk signaling.

The regulation of 4E-BP1 and eIF4F via MEK/Erk signaling may be important for the control of translation by mitogenic signals which do not activate PI 3-kinase/PKB. eIF4E is a proto-oncogene (39–41) and MEK/Erk signaling involves two upstream proto-oncogenes (*ras* and *raf*) so that the activation of

eIF4E, by release of the repressor 4E-BP1, may link the regulation of translation to oncogenic signaling via MEK/Erk.

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The Extracellular Signal-regulated Kinase Pathway Regulates the Phosphorylation of 4E-BP1 at Multiple Sites

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